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TITLE: SIRT3 Is a Mitochondrial Tumor Suppressor and Genetic Loss Results in a Murine Model for ER/PR-Positive Mammary Tumors Connecting Metabolism and Carcinogenesis

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14. ABSTRACT

The goals of this synergistic project are to establish the mitochondria localized sirtuin protein SIRT3 as a tumor suppressor in breast cancer and to define its role as a molecular link between aging and breast cancer. Sirt3 knockout mice develop ER/PR-positive breast tumors later in life. These ER/PR-positive tumors are histologically similar to breast tumors common in older women. In humans, loss of SIRT3 is seen in a significant fraction of breast cancers and may serve as a molecular biomarker. Molecular targets of SIRT3 deacetylation have been identified, including MnSOD and OSCP. Antibodies that recognize specific acetylated lysine residues targeted by SIRT3 in these molecules have been identified and validated and are being developed as potential novel biomarkers in breast cancer. These studies have enhanced our understanding of the molecular links between aging and breast cancer and provide novel potential biomarkers of breast cancer in humans.

15. SUBJECT TERMS

Breast cancer, SIRT3, acetylation, aging, biomarkers

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INTRODUCTION

The mammalian *Sirtuin* genes are homologs of the yeast Saccharomyces cerevisiae *Sir2* gene that is implicated in the regulation of longevity (Haigis et al., 2012). There are seven Sirtuin proteins, with SIRT1, SIRT6, and SIRT7 localized in the nucleus, SIRT2 localized in the cytoplasm and SIRT3, SIRT4, and SIRT5 localized in the mitochondria (Finkel et al., 2009). The mitochondrial deacetylase SIRT3 is thought to act on numerous substrates to regulate seevral processes including fat and amino-acid metabolism as well as electron transport (Huang et al., 2010).

SIRT3 has been proposed to fucntion in maintaining mitochondrial integrity and to serve as a bonafide tumor suppressor (Finley et al., 2011; Kim et al., 2010). We have shown that Sirt3−/− MEFs expressing only Myc or Ras are able to grow in soft agar and form tumors in nude mice, unlike wild type MEFS which require the expression of both oncogenes (Kim et al., 2010). Furthermore, Sirt3 knockout mice develop estrogen receptor and progesterone receptor (ER/PR) positive breast cancers (Kim et al., 2010).

One third of female Sirt3 knockout mice developed mammary gland tumors by 24 months. These tumors were well-differentiated, ER/PR+ tumors similar to the tumors commonly seen in breast malignancies in older women. Our analysis of 992 human breast cancer samples from human tumor mRNA expression databases showed a significant reduction in SIRT3 mRNA in breast cancers compared to benign tissue as well as an association with grade (Kim et al., 2010).

BODY

Statement of Work - Task 1 - Identify Sirt3 mitochondrial deacetylation targets and determine if these targets are regulated by extracellular stimuli known to activate sirtuin function (e.g., resveratrol). These targets will subsequently be knocked down (with siRNA) to determine if there is a mechanistic connection between the increase in superoxide and the stress-induced genomic instability observed in SIRT3$^{-/-}$ cells (months 1-18).

Results: The results for Task 1 have been detailed in the report for W81XWH-10-1-0661, which is the companion grant for W81XWH-10-1-0662 in this synergistic award mechanism. These studies have identified Manganese Superoxide Dismutase (MnSOD) and OSCP as SIRT3 targets. The specific lysine residues in these proteins targeted by Sirt3 have been identified and new antibodies have been developed. These include the OSCP acetylated K139 and MnSOD acetylated K68 antibodies.

Statement of Work - Task 2 - Determine if exposure to resveratrol or overexpression of a MnSOD gene will prevent increases in ROS in MEFs and/or decrease the development of mammary tumors in *Sirt3* knockout mice and transformation in SIRT3$^{-/-}$ MEFs (months 7-24).

Results: The mice for this study are being generated by W81XWH-10-1-0661 while W81XWH-10-1-0662 will be involved in the analysis of the mammary tumors in Sirt3 knockout mice. The animals have been bred and are being aged, since mammary tumors develop in older mice after 1 year of age. These studies were slated to start in month 7 and are ongoing.

Statement of Work - Task 3 - Determine if loss of SIRT3 ductal protein in ER/PR-positive and -negative breast samples from the Vanderbilt Breast Spore correlates with clinically significant outcomes including response to therapy, local tumor control, disease free survival, and overall survival (months 1-24).

We have used a human breast tissue array to determine conditions for SIRT3 staining (Figure 1). We have validated the new antibodies generated against acetylated SIRT3 substrates, including MnSOD-K68 and OSCP-K138. We described in the first year annual report the western blot analyses validation of these antibodies. We next worked to define the conditions for immunohistochemistry using these antibodies. Because human

breast cancer samples available to us are available as formalin-fixed, paraffin-embedded (FFPE) tissue, we sought to work out antibody conditions for staining FFPE tissue sections. We tested antigen retrieval conditions in citrate buffer, pH 6 and EDTA buffer pH9. Figure 2 shows examples of images from successful staining by the new antibodies in FFPE tissues by immunohistochemistry in Sirt3 knockout mouse tissue. We are now validating these findings in human sections before proceeding to analysis of tissue microarrays.

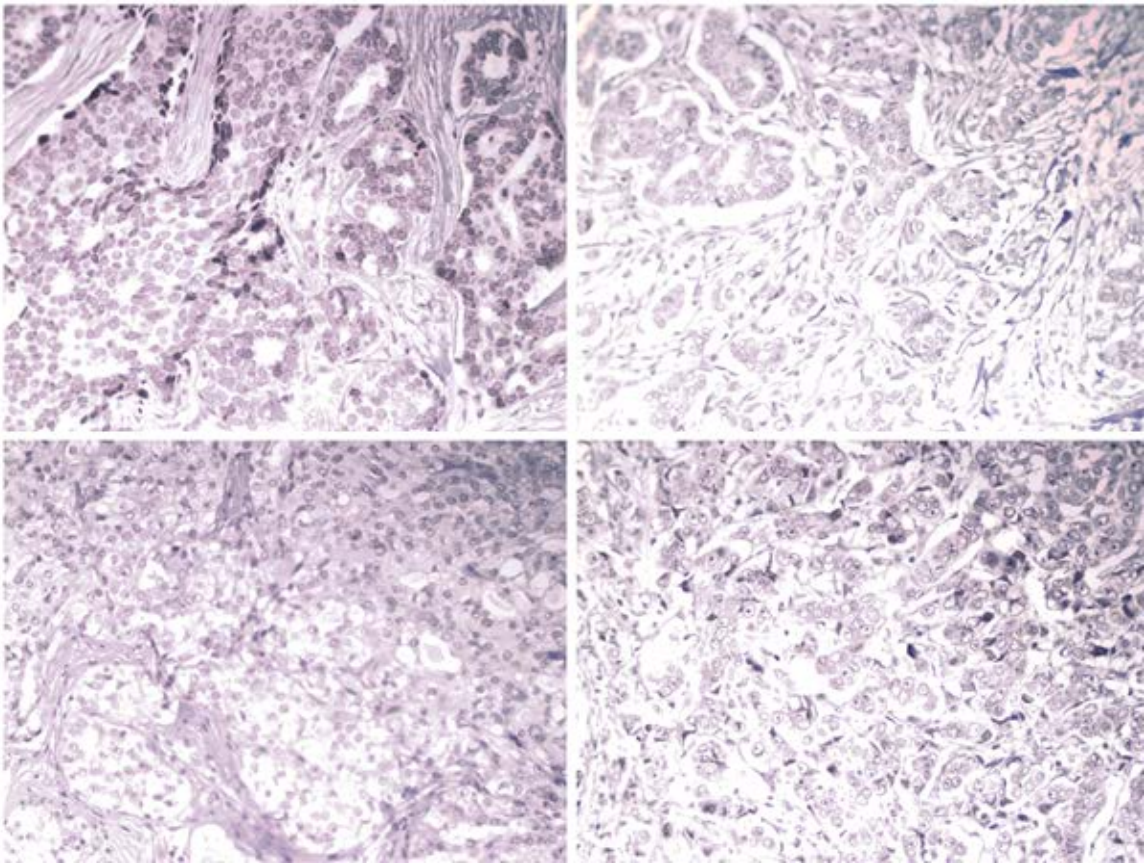

Figure 1. Examples of SIRT3 staining pattern in a breast cancer tissue array, showing various degrees of loss of SIRT3 staining.

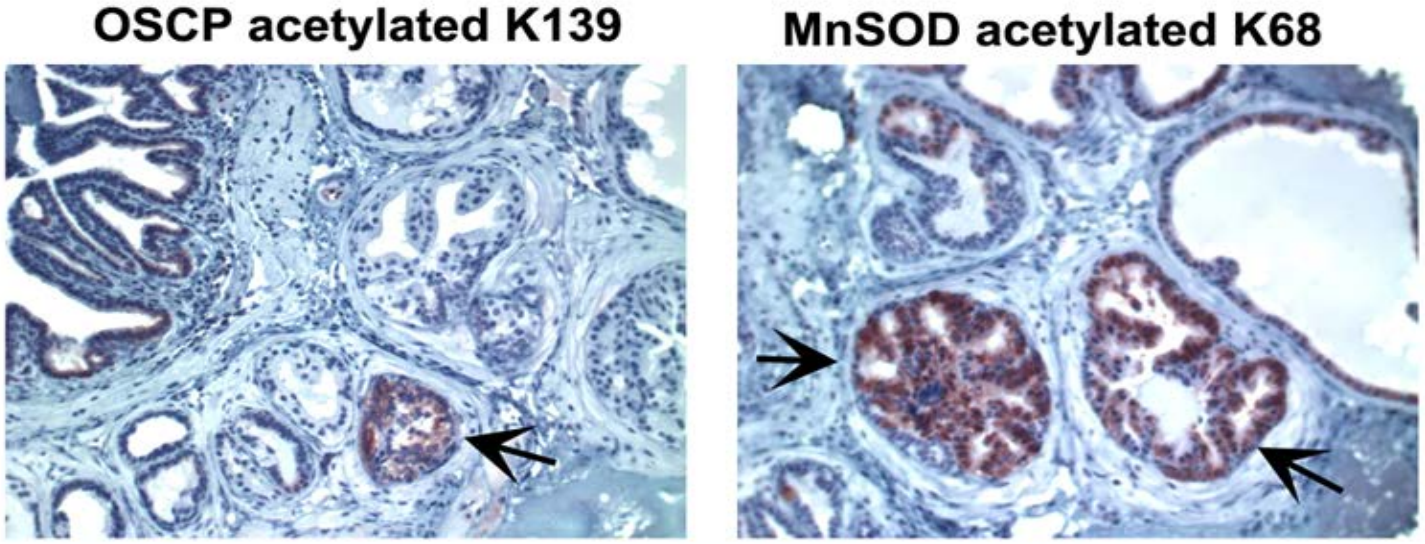

Figure 2. Validation of the OSCP acetylated K139 and MnSOD acetylated K68 antibodies. Sirt3 knockout mouse prostate sections were stained with the indicated antibodies and positive glands showing cytoplasmic staining detected by Nova red staining (arrows). Negative glands on the same sections serve as internal negative control. No primary antibody controls shows no staining (not shown).

This work is on-going and was slightly delayed to accommodate the validation of the new antibodies may be molecular biomarkers. In this task we will determine clinicopathologic variables including age, tumor grade, stage, mitotic count, and ER and PR status and relate these to SIRT3 and its substrates. We will be submitting a no cost extension to allow completion of these studies as outlined in the statement of work.

KEY RESEARCH ACCOMPLISHMENTS:

1. Validation of SIRT3 loss in human breast cancer samples.
2. Validation of antibodies for SIRT3 lysine substrates in MnSOD and OSCP in immunohistochemistry using formalin-fixed paraffin embedded samples.

REPORTABLE OUTCOMES:

1. Antibodies for SIRT3 lysine substrates in MnSOD and OSCP suitable for immunohistochemistry

CONCLUSION:

Aging has long been recognized as a risk factor for breast cancer incidence, but the molecular basis for this association is not understood. Our studies suggest that SIRT3 may provide a molecular link between breast cancer and aging. This synergistic DOD idea award has enabled the development of a Sirt3 knockout mouse model of aging-related receptor-positive breast cancers. In addition, new potential biomarkers for breast cancer are being developed. These studies provide important new insights into breast tumorigenesis as well as provide possible new therapeutic and prognostic targets for human breast cancer.

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